The power of network-based drug design and the interplay between metabolism and gene expression in Trypanosoma brucei

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Summary

For many infectious diseases (mainly for protozoan infections) good medication is lacking, despite regular reports of newly identified drug targets. One of the reasons for this discrepancy may lie in the complexity of living cells, with many interconnecting pathways and signaling routes that are also not static but can be altered by changed expression of constituents. Because of these complex (interactions of) networks, the effect of perturbations to a system is not easy to predict. Consequently, drugs that do strongly affect a molecular target fail to work in the intact human, or in many humans. And many of the drugs that do have an effect in the human host turn out to be toxic for at least a large number of individuals, reflecting that the networks differ between individuals.

To facilitate a more rational, network-based approach to drug design, in the introduction (*Chapter I*) to this thesis we proposed an ordered list of five criteria for good drug targets, namely:

1. *The target pathway should be essential for pathogen survival and/or growth*

2. *The target should exert a high control on pathway output in the pathogen*

3. *The gene-expression response of the pathogen should not counteract the primary inhibition*

4. *The drug should be able to reach the target* in vivo

5. *Side effects should be limited*

While criterion 1 is well-established and an obvious prerequisite for a successful drug target, tackling the other criteria necessitates a quantitative understanding of the pathway in the pathogen (*e.g.* a cancer cell or a parasite) and its host at multiple levels (*e.g.* the pathway level and gene expression)

In the work described in this thesis we have studied glycolysis in *Trypanosoma brucei*. This is a eukaryotic parasite that causes the deadly African sleeping sickness in human, and nagana in cattle. The parasite is transmitted between mammals and the tsetse fly by bites of the latter. *T. brucei* can survive in these two distinct classes of organisms by differentiation into a life stage that is adapted to either host, *e.g.* with respect to metabolism and coat proteins. The bloodstream form of the parasite (which lives extracellularly in the mammalian bloodstream) has glycolysis as its sole source of ATP. Glycolysis thus fulfils the first criterion in our list.

The aim of the work on this thesis consists of two parts. First we want to enhance the quantitative knowledge concerning the behaviour of *T. brucei* glycolysis within the complex networks of the growing parasite. This involves understanding of the steady-state pathway structure, but also of the regulation of the rates of the reactions carried out by the glycolytic enzymes and to which distribution of flux control these lead. This quantitative understanding will contribute to achieve the second aim, the identification and confirmation of potential targets for antitrypanosomal drugs.

To understand the behaviour of a single pathway within a complex and growing organism with many interconnections between pathways one needs a well-defined experimental test system. In recent years, trypanosome research has shifted from the use of non-growing trypanosomes isolated from infected rats to an *in vitro* culture system of growing parasites. In *chapter II* we showed that we can measure reproducibly both growth rate and glycolytic flux in this *in vitro* system. We also showed with ^{13}C-labelled glucose that glycolysis can be studied as an 'isolated' pathway within the trypanosome as the carbon from glucose is almost exclusively converted into pyruvate. Both the glucose consumption and the pyruvate production flux are thus quantitative measures of glycolytic flux and ATP production.

Also in *chapter II*, we updated the previously constructed kinetic computer model [26] of trypanosome glycolysis to this *in vitro* system. We used enzyme activities measured in *in vitro* grown trypanosomes, included two enzymes for which experimental kinetic information had become available and updated the kinetic information for phosphoglycerate kinase (PGK) and glycerol kinase. While flux control distribution (which is a quantitative measure of criterion 2) is largely similar in the new and the old version of the model, the new version gives a better description of the inhibitory effect of glycerol on anaerobic glycolytic flux.

T. brucei glycolysis has an unusual pathway structure. Part of the pathway is compartmentalised inside specialised peroxisomes, called glycosomes. Previous simulations with earlier versions of the model have led to the hypothesis that glycosomes prevent usage of the ATP generated in the final part of glycolysis (in the cytosol) in the reactions catalysed by hexokinase (HXK) and phosphofructokinase (PFK). Removing the glycosome *in silico* resulted in accumulation of phosphorylated glycolytic intermediates in trypanosomes upon addition of glucose, as the parasites lack a negative feedback of such compounds on HXK and PFK. The results in *chapter III* show that this effect is retained in the new version of the model. In addition this chapter serves to demonstrate that *in silico* glycerol catabolism has the same autocatalytic nature as glucose catabolism, such that in the absence of a glycosomal membrane and in the presence of glycerol, glycerol 3-phosphate accumulates. We then show with PEX14 deficient trypanosomes, which are

impaired in glycosomal protein import, that glucose addition indeed leads to accumulation of glucose 6-phosphate and that glycerol administration results in toxic levels of glycerol 3-phosphate. As predicted by the model, depletion of glycerol kinase rescued PEX14-deficient cells of this glycerol toxicity. This provides the first experimental support for our hypothesis that pathway compartmentation is an alternative to allosteric regulation for solving the major problem associated with the usual autocatalytic nature of catabolic pathways, *i.e.* that of a lethal metabolic explosion. Furthermore, this shows the power of computer model predictions and provides additional rationale to the glycosome as a drug target.

As altered gene expression can alter pathways quantitatively (and thereby drug efficacy, necessitating criterion 3), in *chapter IV* we ventured to expand our quantitative analysis methodology to the level of gene expression. We studied the case of PGK, an enzyme of which the trypanosome has three isoforms. The isoforms have a distinct expression pattern, both at the mRNA and at the protein level, with PGKB almost exclusively expressed in the insect (procyclic) stage and PGKC the predominant isoform at the bloodstream stage. As is more general in the transcription of the trypanosome genome (also for non-isoform genes), the PGK isoforms are transcribed in tandem as a long polycistronic precursor messenger [111], which is later spliced to form the mature messengers. This organisation precludes transcription regulation of the levels of individual transcripts at the level of gene transcription. We asked the question which processes control *PGK* mRNA levels at steady-state conditions and at which level of the gene-expression cascade PGK isoform expression is regulated by the differentiating parasite. Our measurements reveal that steady-state *in-vitro*-growing bloodstream-form trypanosomes have low numbers of *PGK* transcripts (on the average12 molecules per cell) but many protein molecules (on the average 10^6 per cell). Furthermore, we determined the rates of splicing and degradation of the precursor mRNA molecules and assessed how many ribosomes occupy a PGK transcript. With this information and the published half lives of the mature transcripts, we built a kinetic model of the PGK expression system. Control analysis of this model revealed that mature transcript levels are positively controlled by transcription and negatively by mRNA degradation, without a substantial quantitative role for splicing or precursor degradation. Yet, when the parasite differentiates from the bloodstream to the procyclic life stage, the isoform mRNA expression is predominantly regulated at the level of mRNA degradation and not by transcription. The quantitative model of PGK gene expression may serve as a mould for the addition of the gene expression level to our metabolic model, and as an example of how this can be done for other metabolic pathways in other organisms. Parameters will probably have to be determined for all enzymes separately.

In 2005, Albert and coworkers [6], published on the effects on growth and metabolism of large decreases in enzyme activities of either HXK, PFK, phosphoglycerate mutase (PGAM), enolase (ENO) or pyruvate kinase (PYK) induced by RNA interference. In *chapter V* we have compared these effects on glycolytic flux to simulations with the new model (described in *chapter II*). For ENO the kinetic model reflects its *in vivo* role. In line with model simulations, HXK and PYK are in excess (more than 50% of wild-type flux remains with only 25% of enzyme activity left), although their overcapacity is overestimated by the model. Depletion of PFK *in vitro* leads to more dramatic effects than we find *in silico*, but depletion of PFK is accompanied by decreases in enzyme activity of other glycolytic enzymes. The fact that not all enzymes were monitored precludes a direct comparison of model and experiment in this case. Finally, the results for PGAM were used in the construction of the model and hence model and experiment show similar results. In all, these results show that, while in this test the model performs reasonably well *qualitatively*, on the *quantitative* levels improvements are needed. Notably, while in the computer model only the activity of one enzyme was altered, the results of Albert *et al*. already indicated that large enzyme changes evoke changes in the expression of other glycolytic enzymes (*e.g.* PFK), which, may in part account for the differences between model and experiment. To further study gene expression changes, in two of these mutants (HXK and PFK) we measured the effects a large decrease in enzyme activity on the mRNA levels of the other glycolytic enzymes (*chapter V*). Importantly, in this analysis we included the glucose transporters, which were missing in the study by Albert *et al.* and which are known to exert a high flux control. We found that indeed mRNA levels for many other enzymes change in response to HXK or PFK depletion, amongst which the glucose transporters. Gene expression changes may play an important role in the regulation of glycolytic flux and might thereby alter the potential of a drug target that had solely been designated based on results obtained from metabolic considerations alone. These results underpinned the possible importance of criterion 3.

Earlier *in vitro* and *in silico* studies [27,28] assigned a high flux control to *T. brucei* glucose transport. Hence, the glucose transporter is the most prominent drug target from a metabolic perspective. However, homeostatic gene expression responses of the parasite to glucose transport inhibition might lower the potential of glucose transport as a drug target (criterion 3) and would necessitate high doses, which in turn may lead to unwanted side effects in the host (criterion 5). To study the response to such inhibition, in *Chapter VI* we inhibited glucose transport in bloodstream-form *T. brucei* with two chemically different inhibitors, phloretin and 2-deoxy-D-glucose. High doses that inhibit glycolytic flux by more than 50% killed the parasite as had been observed in other studies where activities of other glycolytic enzymes had been attenuated. Sublethal doses of glucose transport inhibitors, which halt parasite growth but do not lead to cell death, evoked a distinct gene expression response:

mRNA levels for many glycolytic enzymes, including the bloodstream-form isoform of the glucose transporter, were down regulated, while transcript levels for typical procyclic enzymes were elevated. For citrate synthase, an enzyme that is only expressed in procyclic trypanosomes, activity measurements showed that this change was not confined to the mRNA level. Glucose transport inhibition induced more procyclic features: mRNA for procyclins (procyclic coat proteins) was induced, cells became concanavalin-A sensitive (indicating functional procyclin expression), and cells acquired the ability to survive transition to procyclic culture conditions. In the context of the mammalian host, this onset of a differentiation process and notably the expression of procyclin coat proteins is expected to provide targets for the immune system. These results show that, while one would expect that the parasite changes gene expression to counteract the inhibition, the actual gene expression response observed in this study may actually enhance the potential of glucose transport as a drug target. It will be worthwile to study the gene expression responses to inhibition in other pathogens (a term by which we also mean aberrant cell types such as cancer cells) and focus on the responses that are anti-homeostatic.

In this thesis we have mainly focussed on criteria 2 and 3 of the list postulated in the introduction. The results for these criteria provide the strong targets for which structural drug designers can make compounds that can reach the target (criterion 4) and specifically affect the parasite without harming the host (criterion 5). The glucose transporter has the advantage that it is easily accessible. Furthermore, unpublished results from an *in silico* comparison of glycolysis models of *T. brucei* and the erythrocyte (Bakker *et al.*, in preparation) indicate differences in the flux-control distribution between trypanosome glycolysis and its blood neighbour the erythrocyte that make erythrocytes less vulnerable to glucose transport inhibition than trypanosomes. Considering all these aspects together qualifies glucose transport as a potentially extremely promising drug target.

The results in this thesis show the strength of the combination of *in vitro* and *in silico* tools in quantitative understanding of cellular pathways on multiple levels. Such a quantitative understanding can facilitate rational, network-based drug design.